

MODELLING OF DIACETYL PRODUCTION DURING BEER FERMENTATION

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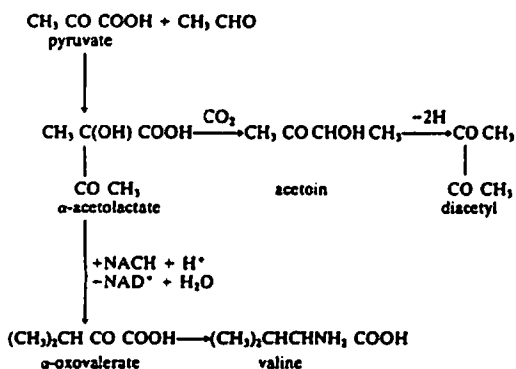
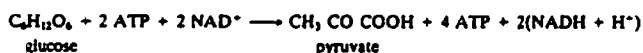
Experimental brewing in stirred tanks has been carried out at different temperatures, pH's and agitation characteristics. A model based on biochemical pathways has been developed. The parameters have been evaluated to fit the results of diacetyl concentration evolution throughout fermentation. The resulting model has proved itself to be useful in predicting results in industrial cylindro-conical fermenters when delay due to non-agitation during the first few days is considered.

INTRODUCTION

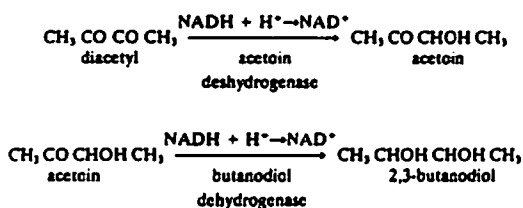
Diacetyl concentration is possibly the best known quality parameter of brewing; one of the most important biotechnological industries from an economic standpoint. To be able to predict its evolution during fermentation, depending on the operation trajectories, is a matter of interest from a bioengineering point of view.

The mechanism of diacetyl synthesis and degradation by yeast is very well known^{1,2,3}. The uptake of amino acids by yeast follows a sequential order, and this uptake of amino acids can be classified into four groups. The amino acid valine, of great importance in the metabolism of diacetyl, is absorbed within the second group. Acetolactate, which is the precursor of the amino acid valine, is formed from glucose, diacetyl also being obtained as a by-product of this reaction. Once diacetyl has been excreted to the medium by the cells, is quickly taken up by these and enzymatically reduced to acetoin and finally to 2-3 butanediol, which is excreted. This last product does not impart any undesirable flavour to the beer in contrast to diacetyl^{1,2,3}. A scheme for these sequence is:

A) Diacetyl production



B) Diacetyl reduction



The generally accepted classification of amino acids into groups depending on their uptake by the yeast is as follows^{4,5,6}: Group A—glutamate, glutamine, aspartate, asparagine, serine, threonine, lysine and arginine. Group B—histidine, valine, methionine, leucine and isoleucine, Group C—glycine, phenylalanine, tyrosine, tryptophan and alanine. Group D—proline and aminobutyric acid.

At the beginning of the fermentation process the group A is immediately absorbed and utilized, meanwhile the group B is also absorbed, but gradually during the whole course of fermentation. When valine (group B) is not absorbed in sufficient quantities, the enzymes that participate in its synthesis are activated resulting in the formation and posterior excretion of the α -acetolactate. When valine is absorbed in sufficient quantity, the synthesis of this amino acid stops as does the excretion of α -acetolactate.

At low temperatures and without external stirring, the group C is not utilized, but, at high temperatures and with some external agitation, it is; in which cases valine is synthesized again as well as diacetyl. The group D is only absorbed by the yeast if the other three groups have already been completely consumed and if oxygen is present at some extent. Once the amino acids content remains constant in the medium, diacetyl concentration diminishes because of reduction by microorganisms.

MATERIALS AND METHODS

The yeast *Saccharomyces carlsbergensis*, (used by "Versuchs- und Lehranstalt für Brauerei in Berlin (VLB)), was an industrial bottom strain employed by the brewery "El Aguila Negra" in Spain. The yeast cells were maintained at 4°C on agar slopes. The microorganism was cultured in a natural wort medium (pH = 5.1; "B" = 11.8). The main components of the wort were analyzed and the following results were obtained: Lipids by Gas-chromatography (G.C.), (Perkin Elmer-8600) equipped with a flame ionization detector (F.I.D.) 1.51 ppm; amino acids by High Performance Liquid Chromatography (H.P.L.C.), (Perkin Elmer-313) equipped with a fluorescence detector 1566.63 ppm; and fermentable sugars by H.P.L.C. (Hewlett-Packard-1050) equipped with a refraction index detector 8.18 g/100 ml.

Cell growth evolution was monitored by measuring dry weight. The evolution of different amino acids was determined by G.C., as indicated. Samples were treated with ethanol 50% in order to precipitate proteins. Afterwards, the amino acids in the supernatant were derivatized in the following way: 0.25 ml of sodium borate, 0.75 ml of methanol and 125 ml of ortho-ftaldialdehyde were added to 0.125 ml of sample. A volume of 0.050 ml was injected into a m-Bondapak C₁₈ 125 Å 10 µm 3.9 × 300 mm. The column was operated at room temperature and the flow rate was of 2 ml/min. the eluants were acetonitrile, acid sodium phosphate 12.5 mM, pH = 7.2, with a linear gradient from 9% to 49% in acetonitrile in 40 minutes. The evolution of vicinal dike-

tones (diacetyl) were tested by the Parnas & Makham method⁷.

Cells were cultivated in two steps as follows: a preculture, grown overnight in the same wort medium at 25°C, in Erlenmeyer flasks plugged with cotton wool (50 ml in 250 ml flasks) and in an incubator shaker (New Brunswick Scientific Co. Inc., N.B.S., Model G25) at 100 r.p.m., inoculated in the culture at 10^7 cells/ml. This preculture was also grown aerobically, by adding air continuously by means of a peristaltic pump.

The cultures at a laboratory level were carried out in a 5 litre laboratory fermenter (N.B.S., BIOFLO III) with external agitation (150 r.p.m.) at the temperatures indicated in each case. The pH was kept constant, if necessary, by automatically adding a solution of NaOH 0.01 mM. The medium was initially saturated with oxygen (9 p.p.m.). In some special cases, cultures were carried out in a similar way to the precultures in 250 ml Erlenmeyer flasks. Cultures were also carried out in a 2×10^5 litre (some 3.5 m of diameter and 18 m of height) industrial, non externally agitated, cylindrico-conical fermenter. In this case, the inoculum was added to the fermenter in several portions at the same time as the wort. The temperature of fermentation was 12°C.

The results presented are always the mean of at least three different assays. Moreover, in the case of fermentations carried out on a laboratory scale experiments were carried out at three independent times. The mean error for the concentration of diacetyl at the different assayed conditions varies between 5 and 8%, being of this order for the rest of the variables assayed with the exception of the different groups of aminoacids, in which the average error reached values of 11%.

RESULTS AND DISCUSSION

Analysis of precursors and previous results

Since the consumption of valine, within the second group of aminoacids, is of interest for the production of diacetyl, the pattern of aminoacids was monitored throughout the fermentation process, both quantitatively and qualitatively. The results obtained for the two types of experiments carried out in small scale bioreactors, with and without external agitation, are represented in Figures 1 and 2. These results are presented by grouping the aminoacids depending on the order in which they are consumed. The data points represent

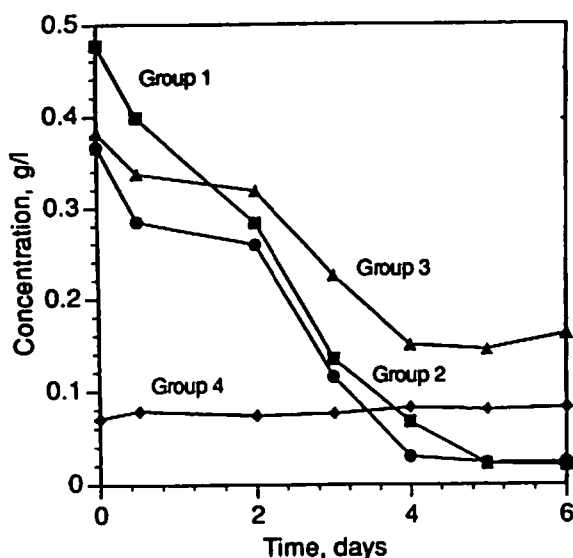


Fig. 2. Absorption of the different groups of aminoacids (group 1: \square ; group 2: \circ ; group 3: \triangle ; group 4: \diamond) during fermentation without external agitation.

a summation of the concentration of aminoacids in each group.

These experiments confirmed that when fermentation starts, group A amino acids are absorbed at a higher rate than groups B and C. Later, group B amino acids start to be absorbed at a higher rate. At the end of the fermentation, with external stirring, all the aminoacids are absorbed, but without stirring, only the fourth group remains not absorbed, with a small amount of the others remaining. Although a small amount of group C is initially absorbed from the beginning of the fermentation process, practically all group 3 is absorbed later, as was earlier reported^{4,5,6}.

When incubation was carried out in Erlenmeyers of 250 ml, with external agitation, two peaks in the diacetyl concentration were observed, Figure 3. The first peak was caused by the rapid assimilation of the group A of amino acids, followed by an enzymatic reduction of the diacetyl at the same time as the group B amino acids were absorbed. The

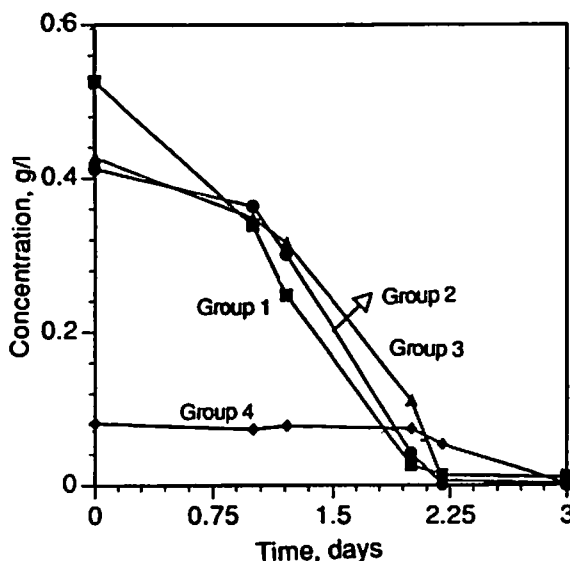


Fig. 1. Absorption of the different groups of aminoacids (group 1: \square ; group 2: \circ ; group 3: \triangle ; group 4: \diamond) during fermentation with external agitation.

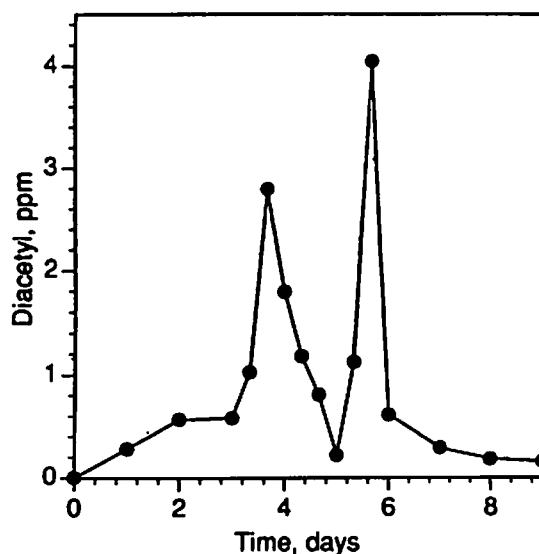


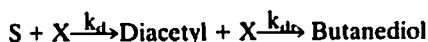
Fig. 3. Production of diacetyl concentration during fermentation in 250 ml flasks with external agitation.

second peak appeared as a consequence of a new synthesis of valine.

Contrary to these results, when incubation took place in a bioreactor with or without external agitation, only one peak of diacetyl was observed. Since diacetyl production is related only to valine synthesis by the cells, in Figure 4 evolution of diacetyl production and valine absorption, as well as the dry weight, are given. In this figure the results obtained in the 5 litre fermenter are supplied since the profiles of the curves are very similar to the industrial reactor.

Kinetic Model

On the basis of the simplified scheme of reactions;



where k_d and k_{dr} are the diacetyl formation and reduction constants. S and X represent the substrate and the cell concentration, respectively. A kinetic equation can be proposed, such as:

$$dC_d/dt = k_d r_s C_x - k_{dr} C_d C_x \quad (1)$$

where C_d and C_x are the diacetyl and the cell concentration, respectively, and r_s is the rate of consumption of substrate.

Nevertheless, it is also necessary to take into account the inhibitory effect that valine absorption presents on the production of diacetyl^{4,5,6}, and the fact that cells lessen their capacity to reduce diacetyl with time. Thus, two different stages can be established: before of valine exhaustion in the medium; and afterwards. For inhibition, it can be proposed:

$$k_d = k_d' [1 + k_v (dC_v/dt)]^{-1} \quad (2)$$

where k_v is the valine formation constant and C_v the valine concentration. When the valine absorption rate increases, the diacetyl formation constant diminishes and hence, its production rate also decreases. Since the reduction rates decrease gradually to zero level, when cell growth stops, a simple empirical relation for the reducing activity effect is proposed:

$$k_{dr} = k_{dr}' t^{-n}; \text{ which in an adimensional form turn into: } \quad (3)$$

$$k_{dr} = k_{dr}'' (t/t')^{-n} \quad (n \text{ is a parameter of the model}) \quad (4)$$

where t' is the time when the diacetyl concentration reaches a maximum (3 days at 12°C).

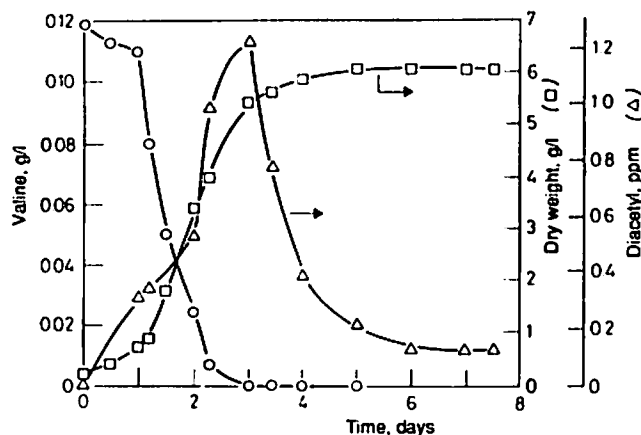


Fig. 4. Production of diacetyl (Δ), valine (\circ) and dry weight (\square) concentration during fermentation with external agitation.

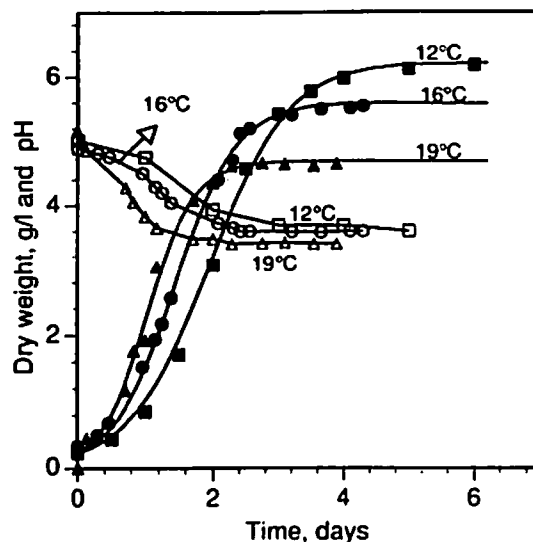


Fig. 5. Dry weight (\bullet) and pH (\circ) evolution during fermentation at different constant temperatures (12°C: squares; 16°C: circles; 19°C: triangles).

Fitting of the model and temperature effect

The influence of temperature on the production and consumption of diacetyl has been previously studied^{8,9}. To study the effect of temperature three different temperatures were used in the fermentation processes: 12°, 16° and 19°C, the pH value, agitation and reactor volume being maintained constant. In Figure 5, the dry weight changes under the same conditions is also presented, to clarify the behaviour of the cells for use in the proposed model. In the same figure, it is possible to see the pH changes for each of the temperatures used. In Figure 6, the diacetyl results are shown in addition to the curves obtained from the aforementioned kinetic equations.

Experimental results agree with aspects mentioned in the literature. Firstly, since diacetyl production and consumption rates increase with temperature; as well as the fact that the final diacetyl concentrations are quite similar in all the cases

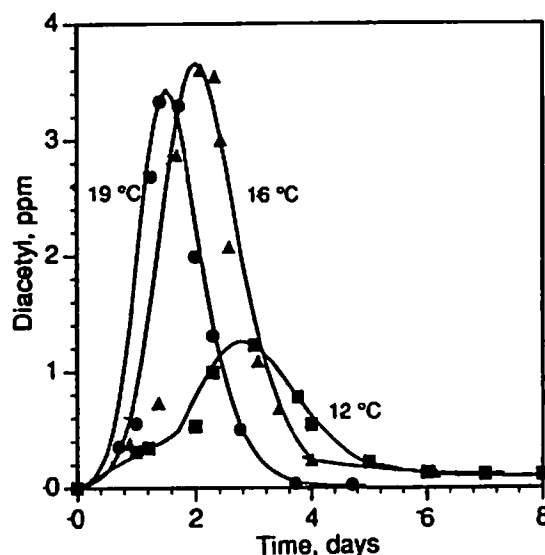


Fig. 6. Experimental (\bullet) and theoretical results (—) for diacetyl rates of production and consumption during fermentation at different constant temperatures (12°C: squares; 16°C: triangles; 19°C: circles).

TABLE I. Diacetyl formation and reduction kinetic parameters

Coefficient	T = 12°C	T = 16°C	T = 19°C
k_d' (l/g)	4.5×10^{-7}	—	—
k_{dr} (l/g h)	4.0×10^{-3}	1.8×10^{-2}	1.9×10^{-2}
k_r (h l/g)	1.0×10^4	—	—
n	4.3	3.2	3.0

examined, 0.110 ppm for 12 & 16°C and 0.022 for 19°C, these small variations may introduce major differences in the flavour of the beer. It is also clear that high temperatures increase the diacetyl reduction process.

As can be seen in Figure 5, the experimental results fit the previously proposed kinetic model well. The values of kinetic coefficients obtained, by a non linear regression program, are given. The constants, which are really average coefficients, for pH; 5.02–3.56, become greater as the temperature increases.

Predictions at Constant pH

The pH effect has already been reported^{10,11}, but in these studies the pH was allowed to develop freely. High pH's always corresponded to high levels of diacetyl. Pajunen¹⁰ *et al.* gave two explanations for this fact: a) Major formation of acetohydroxy acids. (b) Higher acetohydroxy acid conversion rates into diacetyl.

In this work, the pH effect was eliminated by adjusting the pH to a constant values throughout the fermentation process. The chosen values of pH were: the initial pH of the medium, 5.02; the pH at the moment of maximum growth rate, 4.00, and the pH reached at the end of the stationary phase, 3.56. The results obtained are represented in Figure 6 by the three pH's assayed and at a temperatures of 12°C. Figure 7 shows that the pH has a very large effect on the diacetyl reactions of production and reduction. In Figure 8, the dry weight profiles of the yeast are also shown.

At pH 3.56, the cells practically do not grow and the mechanisms previously presented are not the key ones here.

When the pH was 4.00, the profile obtained for diacetyl was quite similar to when the pH was allowed to develop freely. At pH value 5.02, there were two effects: the

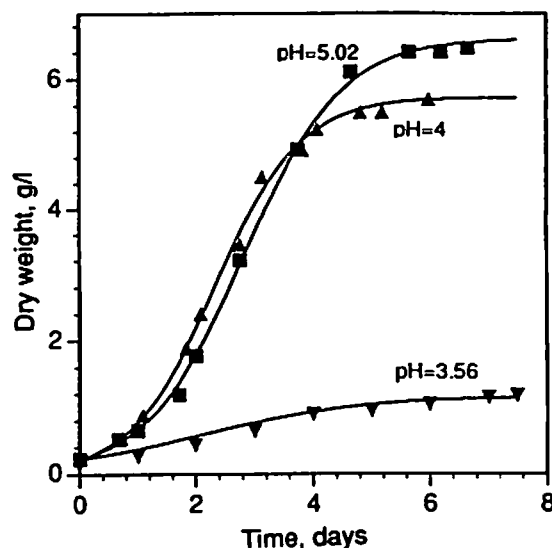


Fig. 8. Dry weight variation during fermentation at different constant pH's (pH 3.56: inverted triangles; pH 4.00: normal triangles; pH 5.02: squares).

maximum concentration reached was very high; and the reduction rate was very low. Therefore, at the end of the process, the residual concentration of diacetyl was very high, 1.55 ppm. Possibly, the reason for the slow reduction rate was the high levels of flocculation observed in the yeast at that time, which implies that sedimentation of the cells occurred. At pH 3.56, the maximum concentration and the reduction rate are both very low. Nevertheless, these two pH's are not of much importance since the original pH of the medium, 5.02, changes very quickly; and at the final pH, 3.56, it is so low that the microorganism hardly grows.

In Figure 9, the comparison at pH 4.00 to the proposed kinetic model is shown. The values of the kinetic coefficients obtained graphically, were the following: k_d' (l/g) = 4.2×10^{-7} ; k_{dr} (l/g h) = 5.0×10^{-2} ; k_r (h l/g) = 1.0×10^4 ; n = 2.1.

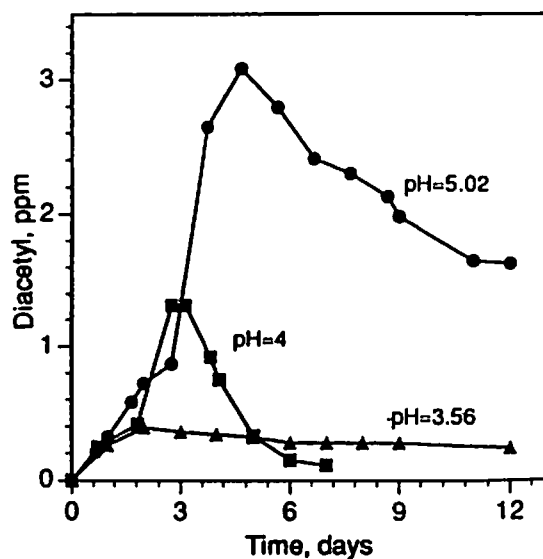


Fig. 7. Diacetyl rates of production and consumption during fermentation at different constant pH's (pH 3.56: triangles; pH 4.00: squares; pH 5.02: circles).

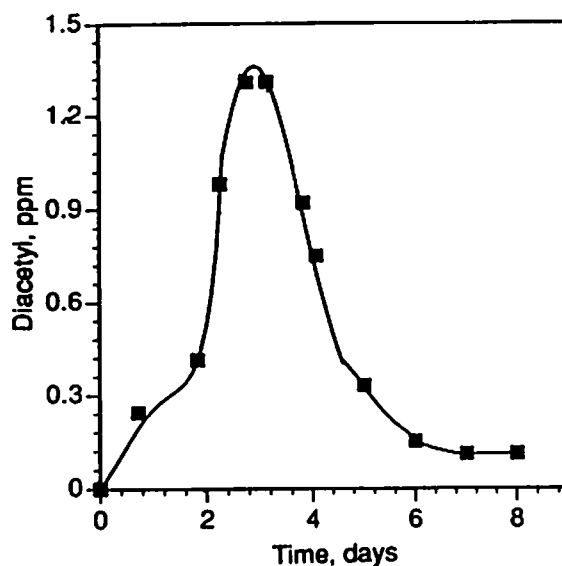


Fig. 9. Experimental (□) and theoretical (—) results of production of diacetyl concentration during fermentation at a constant pH 4.00.

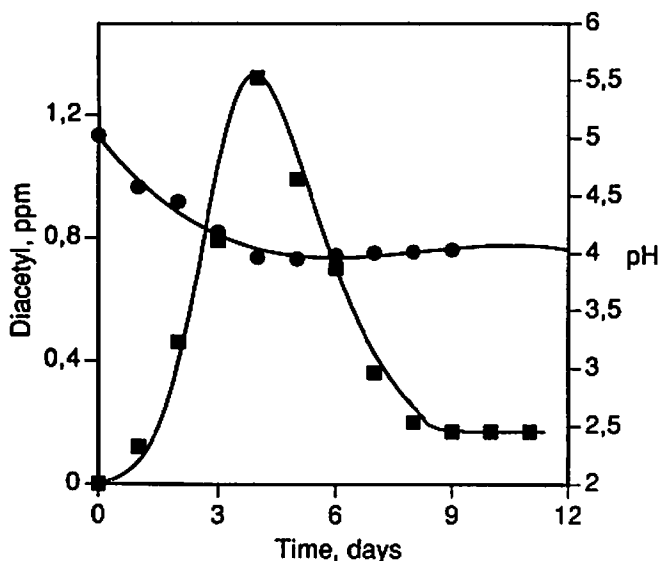


FIG. 10. Production of diacetyl concentration (□) and pH (○) during fermentation at an industrial level.

Application of the model to the industrial plant

Experiments were carried out in a 2×10^5 litre industrial fermenter, under the same initial conditions as those applied to previous experiments and without controlling the pH value. Diacetyl concentration curves obtained in these experiments were found to be quite similar to those described in the previous laboratory results, although a definite delay, τ , in the industrial bioreactor was detected, Figure 10. This is related to the agitation, a high power per unit volume exists only at the high intensity fermentation stage. Therefore, the delay corresponds to the initial absence of agitation in industrial fermentation. The high power always exists in the laboratory stirred tank fermenter. The pH evolution is also shown in Figure 10.

The kinetic equations extracted from the first set of laboratory experiments were modified, taking into account the delay to achieve a model capable of predicting and comparing the behaviour of these compounds in an industrial fermentation. The final equation remains as follows:

$$dC_d/d(t + \tau) = k_d r_s C_x - K_{dr} C_d C_x \quad (5)$$

In the final calculations made, an accurate correlation between the model predictions, with a delay time of 24 hours and the results in the industrial plant is obtained. The analysis

and the good final correlations are useful, not only for diacetyl, but also for predicting the behaviour of other components of interest in industrial fermentations. Moreover, the availability of the kinetic equations to simulate different trajectories of operation, or also for control of the fermentation in plant is demonstrated.

CONCLUSIONS

Diacetyl concentration production throughout the fermentation process can be predicted from a kinetic equation proposed in this work. In this equation, diacetyl concentration is a function of the compounds that participate in its synthesis and degradation: fermentable sugars; the amino acid valine; the diacetyl itself; and the yeast concentration. When fermentation is carried out in flasks with external agitation, the diacetyl production curve presents two peaks instead of one, as in other cases.

Temperature exerts a positive influence on the diacetyl production and consumption rates in the range of temperatures assayed. Experiments at constant and variable pH's can be modelled according to the proposed mechanisms at pH 4.00; whereas the limits of operation, pH 3.56 and 5.01, other considerations may be necessary. Diacetyl production throughout the industrial beer fermentation process can be predicted from the final kinetic equation proposed in this work.

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